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Characterization of Diverse Subvariants of the Meningococcal Factor H (fH) Binding Protein for Their Ability To Bind fH, To Mediate Serum Resistance, and To Induce Bactericidal Antibodies[▽]

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Neisseria meningitidis **is a commensal of the human nasopharynx but is also a major cause of septicemia and meningitis. The meningococcal factor H binding protein (fHbp) binds human factor H (fH), enabling down-regulation of complement activation on the bacterial surface. fHbp is a component of two serogroup B meningococcal vaccines currently in clinical development. Here we characterize 12 fHbp subvariants for their level of surface exposure and ability to bind fH, to mediate serum resistance, and to induce bactericidal antibodies. Flow cytometry and Western analysis revealed that all strains examined expressed fHbp on their surface to different extents and bound fH in an fHbp-dependent manner. However, differences in fH binding did not always correlate with the level of fHbp expression, indicating that this is not the only factor affecting the amount of fH bound. To overcome the issue of strain variability in fHbp expression, the MC58Δ*fHbp* strain was genetically engineered to express different subvariants from a constitutive heterologous promoter. These recombinant strains were characterized for fH binding, and the data confirmed that each subvariant binds different levels of fH. Surface plasmon resonance revealed differences in the stability of the fHbp-fH complexes that ranged over 2 orders of magnitude, indicating that differences in residues between and within variant groups can influence fH binding. Interestingly, the level of survival in human sera of recombinant MC58 strains expressing diverse subvariants did not correlate with the level of fH binding, suggesting that the interaction of fHbp with fH is not the only function of fHbp that influences serum resistance. Furthermore, cross-reactive bactericidal activity was seen within each variant group, although the degree of activity varied, suggesting that amino acid differences within each variant group influence the bactericidal antibody response.**

Diseases caused by *Neisseria meningitidis*, including meningococcal septicemia and meningitis, are a significant health problem worldwide, and their control is largely dependent on the availability and widespread use of vaccines (44). The majority of meningococcal disease is caused by five serogroups, and effective capsular polysaccharide and conjugate vaccines are available to prevent disease caused by serogroups A, C, W-135, and Y (reviewed in reference 52). However, there is still no broadly protective vaccine to combat serogroup B (MenB) disease, which is the main cause of meningococcal disease in the developed world (35, 43). The capsule polysaccharide of MenB is highly similar to glycoproteins in human neural tissues and as such is poorly immunogenic and an unsuitable vaccine candidate (9, 32). However, several outer membrane proteins have been identified as potential antigens for a MenB vaccine (reviewed in reference 23).

Factor H binding protein (fHbp) is a meningococcal protein antigen that is currently in vaccine development (previously referred to as GNA1870 [11] and LP2086 [10]). fHbp is a *Neisseria*-specific 29-kDa surface lipoprotein that binds human factor H (fH), a key inhibitor of the complement alternative pathway (14, 25). fH is a cofactor for the factor I-mediated cleavage and inactivation of C3b and also promotes the decay of the alternative pathway C3 convertase C3bBb (reviewed in reference 29). Therefore, binding of fH to fHbp on the meningococcal surface allows the pathogen to evade complement-mediated killing by the innate immune system. As such, fHbp is important for survival of bacteria in human serum and blood (25, 49, 55). The discovery that binding of fH to *N. meningitidis* is specific for human fH has significant implications for the study of this organism and its species specificity (14). fHbp induces high levels of bactericidal antibodies in mice (11) and humans (4a, 12, 18, 39, 41), and these antibodies activate bacterial killing by the classical complement pathway. Furthermore, it has been shown that binding of antibodies to fHbp can block binding of factor H, thus increasing the susceptibility of the bacterium to killing by the alternative complement pathway (25). Several other microorganisms have evolved with the ability to bind fH and other complement inhibitors in order to evade complement-mediated killing, and many of these proteins have also been studied for vaccine development (29).

fHbp is expressed by almost all *N. meningitidis* strains studied to date (10, 26, 31), although levels of expression vary

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TABLE 1. Meningococcal strains used in this study[a]

Strain	Clonal complex	ST	Yr	Country	Serogroup:serotype:serosubtype	fHbp variant
MC58	32	74	1985	UK	B:15:P1.7,16b	1.1
M01-0240149	41/44	41	2001	UK	B:4:P1.7,4	1.4
M01-0240185	11	11	2001	UK	B:2a:P1.5,10	1.10
NZ98/254	41/44	42	1998	New Zealand	B:4:P1.4	1.14
M1573	41/44	44	1995	USA	B:P7-1,1	1.55
NL096	41/44	110	1960	Netherlands	B:14:NT	1-2,3.x
961-5945	8	153	1996	Australia	B:2b:P1.21,16	2.16
M3153	41/44	5906	1996	USA	B:4,7:P1.4	2.19
C11	NA	345	1965	Cuba	C:NT:P1.1	2.22
M2552	103	103	1996	USA	B:NT:NT	2.25
M1239	41/44	437	1994	USA	B:14:P1.23,14	3.28
M01-0240320	213	213	2001	UK	B:1:P1.22,14	3.45

[a] The fHbps are named in terms of the translated (protein) sequence, as variant class.protein ID, in accordance with the public fHbp database (http://neisseria.org), in which new protein subvariants are assigned a sequential numerical identifier, alongside a prefix corresponding to the Novartis variant designation (variant 1, 2, or 3). For example, fHbp 1.1 refers to Novartis variant 1, neisseria.org protein subvariant 1. *Neisseria* strain NL096 expresses a hybrid natural chimera of fHbp, hence, the designation 1-2,3.x (the x indicates that the peptide number has not yet been assigned). ST, sequence type as determined by MLST; NA, not assigned.

between isolates (26, 28). Furthermore, amino acid sequence diversity exists for fHbp between strains. Several designation methods have been used to characterize different variants, which have been found to be antigenically poorly cross-reactive. Three variant groups, fHbp-1, fHbp-2, and fHbp-3, have been described, where fHbp-1 is the most abundant among group B meningococcus (4), and subvariant 1.1 (fHbp-1.1) is present in the multivalent Novartis MenB vaccine (4CMenB) currently in phase III clinical trials (1, 11, 41). Another system divides fHbp variants into subfamilies A and B, and the bivalent Pfizer (previously Wyeth) MenB vaccine that is in phase II clinical trials contains a representative of each of these families, A05 (subvariant 3.45) and B01 (subvariant 1.55) (10, 20, 28, 31). Subfamilies A and B correspond to variants 2/3 and 1, respectively. A modular architecture has also been described for fHbp that describes the overall architecture of fHbp as being comprised of combinations of five modular variable segments that are flanked by invariable residues, with each of the modular variable segments being derived from either variant 1 or 3 fHbp genes (2).

The purpose of this study was to analyze a selection of the most frequently isolated fHbp subvariants to gain a better understanding of the abilities of different subvariants to bind fH, to mediate serum resistance, and to induce cross-protective bactericidal antibodies.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *N. meningitidis* strains used in this study are described in Table 1. *N. meningitidis* strains were routinely grown on chocolate agar (Biomerieaux), GC (Difco) agar supplemented with Kellogg's supplement I, or on Mueller-Hinton (MH) agar (Difco) at 37°C, 5% CO_2 overnight. For liquid cultures, colonies from overnight growth were used to inoculate 7-ml cultures (in MH broth supplemented with 0.25% glucose) to an optical density at 600 nm (OD_{600}) of ~0.05. The culture was incubated for approximately 1.5 to 2.5 h at 37°C with shaking until early log (OD_{600} of ~0.25) or mid-log phase (OD_{600} of ~0.5). When required, erythromycin and chloramphenicol were used at final concentrations of 5 μg/ml. *Escherichia coli* strains used for cloning were cultured in Luria-Bertani (LB) broth or on LB agar (Difco). When required, ampicillin, erythromycin, and chloramphenicol were used at final concentrations of 100, 100, and 20 μg/ml, respectively.

Phylogenetic analysis and reconstruction of fHbp subvariants. The *fHbp* gene was amplified and sequenced from different *Neisseria* strains as previously reported (1). fHbp amino acid sequences were aligned using MUSCLE v3.6 (8), with default parameters. The evolutionary distances between pairs of aligned fHbp sequences were computed using the JTT matrix-based method (21), units were scaled as the number of amino acid substitutions per site, and standard errors of distances were computed by bootstrap analysis using 500 replicates. The phylogenetic reconstruction based on this distance matrix was inferred using the neighbor-joining method (45). The tree branch lengths are proportional to the evolutionary distances used to infer the phylogenetic tree. All sequence alignment positions containing gaps were eliminated in pairwise sequence comparisons. Phylogenetic analyses were conducted using MEGA4 (53). The amino acid numbering used starts from the first residue (cysteine) of the mature protein.

Cloning and expression of fHbp subvariants in *E. coli*. Recombinant DNA techniques were routinely performed, as described by Sambrook et al. (46). Plasmid DNA preparations and purification of DNA fragments from PCR samples were performed using Qiagen kits according to the manufacturer's instructions. All restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs or Roche. The recombinant proteins were obtained by cloning each gene into the pET-21b+ expression vector (Novagen) and expressing them as C-terminal histidine fusions in an *E. coli* heterologous system [*E. coli* DH5α for cloning and BL21(DE_3) (Invitrogen) for expression].

fHbp genes of subvariants were amplified from the corresponding strains shown in Table 1. Primers 741v1f and 741v1r were used to amplify *fHbp* subvariant 1 genes (except for subvariant 1.55, primers 741v1.55f and 741v1.55r were used and for subvariant 1.4 primers 741v2,3f and 741v1r were used), primers 741v2,3f and 741v2r for subvariant 2 genes, primers 741v2,3f and 741v3r for subvariant 3.28, primers 741v3.13f and 741v3.13r for 3.45, and primers 741v1f and 741v2r for 1-2,3.x (Table 2). The expressed proteins did not contain the leader peptide. For subvariants 1.4, 2.16, 2.19, 2.22, 2.25, and 3.28, the sequence GPDSDRLQQRRG from the gonococcal fHbp homologue was added to the N terminal to aid expression in *E. coli.* PCR conditions used to amplify subvariants were as follows: for 1.1 and 1-2,3.x, five cycles of 94°C for 30 s, 57°C for 30 s, and 68°C for 1 min, then 30 cycles of 94°C for 30 s, 68°C for 30 s, and 68°C for 1 min; for 2.16 and 3.28, 5 cycles of 94°C for 30 s, 56°C for 30 s, and 68°C for 1 min, then 30 cycles of 94°C for 30 s, 71°C for 30 s, and 68°C for 1 min; for the remaining subvariants, 94°C for 2 min, 5 cycles of 94°C for 30 s, 52°C for 30 s, and 68°C for 1 min, then 30 cycles of 94°C for 30 s, 65°C for 30 s, and 68°C for 1 min, and then 68°C 10 min. PCRs were performed with approximately 10 ng of chromosomal DNA using High Fidelity *Taq* DNA polymerase (Invitrogen) as per the manufacturer's instructions. PCR products were digested with NdeI as well as XhoI or HindIII and then cloned into the NdeI/XhoI and NdeI/HindIII sites of pET-21b+. Recombinant plasmids were transformed into the *E. coli* expression strain. Recombinant strains were grown at 37°C to an OD_{600} of 0.6 to 0.8, and expression of recombinant proteins was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma).

Purification of fHbp subvariants in *E. coli*. Bacterial pellets were resuspended in 10 ml of B-PER (bacterial-protein extraction reagent) containing 10 μl of $MgCl_2$ 100 mM, 50 μl of DNase (Sigma), and 100 μl of lysozyme (Sigma), incubated for 40 min at room temperature, and then centrifuged at 35,000 × *g* for 30 min. The supernatant was collected and subjected to two serial purification steps using metal affinity chromatography (IMAC) and ionic exchange chromatography with a desalting step in between. All purification steps were performed using an AKTAxpress chromatographic system, and the OD_{280} was monitored.

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence[a]	Site(s)[b]
U741-F1	gctctagaCCAGCCACGGCGCATAC	XbaI
U741-R1	tccccgggGACGGCATTTTGTTTACAGG	SmaI
D741-F1	tccccgggCGCCAAGCAATAACCATTG	SmaI
D741-R1	cccgctcgagCAGCGTATCGAACCATGC	XhoI
741-F2	ggattccatatgGTGAATCGAACTGCCTTC	NdeI
741-R2	ccaatgcatTTATTGCTTGGCGGCAAG	NsiI
EP1For1.1	CGCggatcccatatgGTGAATCGAACTGCCTTC	BamHI, NdeI
EP5RV1.4	TGCATGCATTTACTGCTTGGCGGCAAG	NsiI
EP2For1.4	CGCggatcccatatgGTGAACCGAACTGCCTTC	BamHI, NdeI
EP6RV2.1	TGCATGCATCTACTGTTTGCCGGCGAT	NsiI
741v1f	cgcggatcccatatgGTCGCCGCCGACATCG	NdeI
741v1r	cccgctcgagTTGCTTGGCGGCAAGGC	XhoI
741v1.55f	cgcggatcccatatgAGCAGCGGAGGCGGCGG	NdeI
741v1.55r	cccgctcgagCTGCTTGGCGGCAAGACC	XhoI
741v2,3f	cgcggatcccatatg*GGCCCTGATTCTGACCGCCTGCAGC AGCGGAGG*GTCGCCGCCGACATCGG	NdeI
741v2r	cccgctcgagCTGTTTGCCGGCGATGCC	XhoI
741v3r	gcccaagcttCTGTTTGCCGGCGATGCC	HindIII
741v3.13f	cgcggatcccatatgAGCAGCGGAAGCGGAAGC	NdeI
741v3.13r	cccgctcgagCTGTTTGCCGGCGATGCC	XhoI

[a] Capital letters correspond to nucleotides of the meningococcal *fHbp* sequence (the gonococcus sequence moiety is reported in italics), and small letters correspond to nucleotides added for cloning reasons (underlined letters indicate sequences of restriction enzyme sites used for cloning PCR fragments).
[b] Enzymes for which the restriction sites are present in the sequence of the primer, added for cloning reasons.

For the IMAC purification step, filtered supernatants were automatically injected into 1-ml Ni^{2+}-HisTrap FF columns with a flow rate of 1 ml/min, and columns were washed with 20 column volumes (CV) of washing buffer (50 mM NaH_2PO_4 [Sigma], 300 mM NaCl [Fluka], 30 mM imidazole [Merck]; pH 8.0). Then, the His tag fusion proteins were eluted with 5 CV of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole; pH 8.0), and automatically loaded on three 5-ml HiTrap (GE) desalting columns connected in series and eluted with a flow rate of 5 ml/min. For ionic exchange chromatography, the eluted proteins were automatically loaded on 1-ml HiTrap Q HP columns with a flow rate of 1 ml/min. Subsequently, the column was washed with 10 CV of 50 mM Tris-HCl, pH 8.0. The elution was set up in a linear gradient, between 50 mM Tris-HCl (pH 8.0) and 50 mM Tris-HCl, 1.0 M NaCl (pH 8.0) buffer in 10 CV, and 1-ml fractions were collected. Protein purity was checked by SDS-PAGE, and the protein concentration was estimated using a Bradford assay.

Mouse immunizations. To prepare anti-fHbp polyclonal antisera, 20 μg of each recombinant protein was used to immunize eight mice (6-week-old CD1 female mice; Charles River). The recombinant proteins were administered intraperitoneally on days 1, 21, and 35. Prior to immunization, fHbp subvariants (100 μg/ml protein) were adsorbed onto aluminum hydroxide (Alum; 3 mg/ml) in 10 mM histidine buffer, pH 6.5 (Sigma) with NaCl (final osmolarity of 0.308/kg) for bactericidal assays. The solution was incubated for 15 min with stirring at room temperature and then stored overnight at 4°C. For antibodies used in flow cytometry, Freund's complete adjuvant was added to the antigen on the day of immunization. Final formulations were isotonic and at physiological pH. Blood samples for analysis were taken on day 49. The treatments were performed in accordance with internal animal ethical committee and institutional guidelines.

Flow cytometry analysis of fH binding and fHbp expression. The ability of meningococci to bind fH was determined using a FACScan flow cytometer. Briefly, approximately 1×10^8 bacteria (grown in MH broth plus 0.25% glucose for approximately 2.5 h at 37°C with shaking until mid-log phase [OD_{600} of ~0.5]) were suspended in phosphate-buffered saline (PBS) plus 1% bovine serum albumin and incubated with purified human fH (whole molecule; Calbiochem), in the quantity specified in each experiment in a final reaction volume of 100 μl, for 20 min at 37°C. fH binding was detected using polyclonal goat anti-human fH antibodies (Calbiochem) and donkey anti-goat IgG–fluorescein isothiocyanate (FITC) conjugate (Jackson Immunoresearch). For competitive inhibition analysis of fH binding, mouse polyclonal antisera specific for each fHbp subvariant (at a 1:100 dilution) were incubated with bacteria for 10 min at 37°C prior to addition of fH.

The ability of mouse polyclonal anti-fHbp sera to bind to the surface of meningococci was measured using a 1:100 dilution of mouse polyclonal anti-fHbp antiserum specific for each subvariant. Primary antibody binding was detected using an anti-mouse (whole-molecule) FITC-conjugated antibody (Sigma) at a 1:100 dilution.

Immobilization of fH on a CM5 chip and kinetic analysis of fH binding by fHbp subvariants. Surface plasmon resonance (SPR) analyses were performed using a Biacore X100 instrument (GE Healthcare). fH was coupled to a CM5 sensor chip (GE Healthcare) in two steps to modulate the total resonance units (RU) of immobilization, using the Biacore amine coupling kit (GE Healthcare). In the first step, the carboxymethylated CM5 dextran layer was activated by a 1:1 (vol/vol) mixture of 0.4 M *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDC) and 0.1 M *N*-hydroxysuccinimide (NHS) in MilliQ water with a 7-min injection time at 10 μl/min. After surface activation, 8 μg/ml fH in 10 mM sodium acetate buffer (pH 4.0) was injected in flow cell 2 with a 9-min pulse and a flow rate of 10 μl/min, reaching 925 RU of protein immobilization. In the second step, 10 μg/ml fH was injected, as described above, to obtain 1,500 RU of immobilized fH. The free *N*-hydroxysuccinimide ester groups were blocked with three manual injections (240 s each) of 1.0 M ethanolamine hydrochloride, pH 8.5. Untreated flow cell 1 was used as a reference. Kinetic analyses were run as follows: 2-fold dilutions, in running buffer (PBS, pH 7.2) of each protein in a concentration range from 15.6 to 500 nM, were injected for 2 min at a flow rate of 45 μl/min followed by a 10-min dissociation time with PBS at the same flow rate. Biosensor regeneration was performed using 100 mM glycine, 3 M NaCl, pH 2.0, with 1-min contact time and a 10-μl/min flow rate. Interaction parameters (k_a, k_d, K_D) were determined by a simultaneous local fitting with a model of equimolar stoichiometry using the BIAevaluation X100 software version 1.0 (GE Healthcare).

Construction of fHbp knockout and complemented strains of *N. meningitidis*. The MC58Δ*fHbp*, 961-5945Δ*fHbp*, and M1239Δ*fHbp* mutant strains have previously been described (formerly named Δ*gna1870*) (26). Complementation of the MC58Δ*fHbp* mutant with different fHbp variants was achieved by insertion of the *fHbp* gene, under the control of the constitutively active P_{tac} promoter, into a noncoding chromosomal location between the two converging open reading frames (ORFs) NMB1428 and NMB1429, as previously described (49). The *fHbp* gene was amplified from the indicated strains in Table 1 (using primers 741-F2 and 741-R2 for 1.1, EP1For1.1 and EP5RV1.4 for 1.10, EP2For1.4 and EP6RV2.1 for 2.16 and 2.25, and EP1For1.1 and EP6RV2.1 for 3.28 [Table 2]) and cloned as a NdeI-NsiI fragment into the $pComP_{KBS}$ plasmid (49). This plasmid was linearized with SpeI and used to transform MC58Δ*fHbp*. Transformants were selected on chloramphenicol and checked by PCR, and complementation of the mutant strain was verified by flow cytometry. Several phase-variable structures were analyzed, and no differences were identified in these strains for lipooligosaccharide (LOS; controlled by silver staining of LOS preparations, as previously described [19]) and Opc (controlled by sequencing of the repetitive DNA tracts associated with *opc*, using previously described primers [47]).

***Ex vivo* human serum models of meningococcal infection.** Bacteria were grown in MH broth plus 0.25% glucose and 0.02 mM cytidine-5′-monophospho-*N*-acetylneuraminic acid sodium salt (CMP-NANA) for approximately 2.5 h at 37°C with shaking until mid-log phase (OD_{600} of ~0. 5) and then diluted in MH broth to approximately 10^5 CFU/ml. The assay was started by addition of 90 μl serum to 10 μl of the bacterial suspension. A sample from this mix was taken immediately and used as time zero. Cultures were incubated at 37°C with gentle agitation, and at various time points an aliquot of the sample was removed and the number of viable CFU was determined by plating serial dilutions onto MH agar. Experiments were performed in triplicate on several occasions. The Student *t* test was used to determine the statistical significance of survival of each complemented strain with respect to either the isogenic wild-type strain or the *fHbp* mutant, with a *P* value of <0.05 considered significant. For preparation of human serum, whole blood was collected from healthy individuals (unimmunized against *N. meningitidis* and with no history of disease), coagulated at 25°C for 30 min, and centrifuged at 1,000 × *g* for 10 min at 4°C, and the supernatant was retained.

Complement-mediated bactericidal activity. Serum bactericidal activity against *N. meningitidis* strains was evaluated as previously described (13) with pooled baby rabbit serum (Pel-Freeze) or human complement obtained from volunteer donors under informed consent (3). Complement donations were screened before use to ensure they lacked endogenous bactericidal activity at concentrations of both 25% and 50% in the assay and that they supported bactericidal activity. Bacteria were grown in MH broth plus 0.25% glucose for approximately 1.5 h at 37°C with shaking until early log phase (OD_{600} of ~0.25) and then diluted in MH broth to approximately 10^5 CFU/ml. Serum bactericidal titers were defined as the serum dilution resulting in a 50% decrease in the CFU/ml after 60 min of incubation of bacteria with the reaction mixture, compared to the control CFU/ml at time zero. Typically, bacteria incubated with the negative-control antibody in the presence of complement showed a 150 to 200% increase in CFU/ml during the 60-min incubation. The titers reported for the sera for anti-fHbp 1.1, 2.16, and 3.28 are averages of four different experiments.

RESULTS

Characterization and phylogenetic analysis of fHbp subvariants. Sequence analysis of fHbp in a worldwide collection of meningococcus B clinical isolates has allowed the identification of more than 330 peptide subvariants (see the Neisseria Multi Locus Sequence Typing [MLST] website [http://pubmlst.org/neisseria/fHbp/]). A set of 12 diverse fHbp subvariants were selected in order to characterize the influence of fHbp sequence diversity on surface expression and the ability of the protein to bind human factor H (fH) and to induce cross-bactericidal antibodies (Table 1). These 12 subvariants were identified in strains belonging to different clonal complexes and sequence types and isolated in different geographic regions in different years, and they included members of each of the three variant groups. The subvariants most frequently found on the basis of variability studies of different strain panels worldwide were included in the analysis (subvariants 1.1, 1.4, 1.14, 2.16, and 2.19) (1, 17, 24). The nomenclature used in this study is in accordance with that used in the public fHbp database (http://neisseria.org) (4), in which new protein subvariants are assigned a sequential numerical identifier alongside a prefix corresponding to the Novartis variant designation (variant 1, 2, or 3) (26), e.g., fHbp 1.1 refers to Novartis variant 1, Neisseria.org protein subvariant 1.

The phylogenetic tree constructed with the amino acid sequences of the 12 fHbp subvariants used in this study (Fig. 1A) resembles the phylogenetic tree already described for the three-variant classification system. One exception is fHbp 1-2,3.x, which is intermediate between fHbp-1 and fHbp variants 2 and 3. The alignment of the amino acid sequences of the 12 fHbp subvariants analyzed (Fig. 1B) showed the chimeric nature of some fHbp subvariants. The fHbp-2 forms were previously described by Beernink et al. (2) as possible chimeric proteins between fHbp-1 and fHbp-3, based on a different classification of five modular variable segments that are derived from either a variant 1 or variant 3 *fHbp* gene. In fact, the sequence alignment highlights that the fHbp-2 subvariants are composed of an fHbp-1 N terminal (residues 1 to 97) and a fHbp-3 C terminal (residues 98 to the stop codon). However, in this paper we refer to fHbp 1-2,3.x as a chimeric form between fHbp-1 and variants 2 and 3, since its N terminal (from residues 1 to 191) is more similar to proteins of variant group 1 (96% identity to 1.1 in this region) and its C terminal (from residue 192 to the stop codon) is more similar to variant groups 2 and 3 (95% identity to both 2.16 and 3.28 and 56% identity to 1.1 in this region). fHbp 1.55 can also be considered another chimeric form, with the N terminal (from residues 1 to 49) containing a group of amino acids typical of variant 3 molecules (96% identity to 3.28 and 80% identity to 1.1 in this region), while the C terminal (from residues 50 to the stop codon) is more similar to the variant 1 group (90% identity to 1.1). Due to the higher homology to the variant 1 group, 1.55 clusters with the variant 1 group. In general, while amino acid variations are scattered along the entire fHbp sequences, higher diversity is seen in the C-terminal part of the molecule, where the majority of the bactericidal epitopes are located (13).

***N. meningitidis* strains carrying different fHbp subvariants differ in their level of surface expression and fH binding.** The level of expression and surface exposure of each fHbp subvariant in the panel of selected strains was evaluated by flow cytometry analysis using mouse polyclonal fHbp homologous antisera (i.e., antisera raised against each subvariant). As shown in Fig. 2A, all strains expressed fHbp on their surface regardless of the subvariant, but the level of expression varied between strains and can be considered high (1.1, 1-2,3.x, 2.19, and 3.28), medium (1.14, 1.55, 2.16, 2.22, 2.25, and 3.45), or low (1.4 and 1.10) for the subvariants expressed by this panel of strains on the basis of fluorescence units of $\geq 10^2$ (high/medium) or $<10^2$ (low). Similar variability of fHbp expression has been described previously (26, 28). Expression of each subvariant in the different strains was confirmed by Western blot analysis of meningococcal whole-cell extracts (data not shown).

Binding of human fH to the surface of each of the meningococcal strains was assessed by flow cytometry using anti-fH antibodies (Fig. 2B). All 12 strains studied bound fH at the highest concentration of fH used (50 μg/ml). By titrating the fH concentration used (5 and 0.5 μg/ml), differences in binding were detected, suggesting that each subvariant has a different affinity for fH. Using 0.5 μg/ml fH, the highest level of binding was seen for strains carrying subvariants 1.1, 2.16, 2.19, and 3.45 and relatively low binding was observed for strains carrying subvariants 1.55, 1-2,3.x, 2.25, and 3.28, while no binding was seen for strains carrying subvariants 1.4, 1.10, 1.14, and 2.22 (Fig. 2B). No fH binding was observed when the analysis was performed using the three *fHbp* knockout mutants, MC58Δ*fHbp*, 961-5945Δ*fHbp*, and M1239Δ*fHbp*, which were used as controls for each of the three variant groups, indicating that for these strains the majority of fH binding is due to fHbp.

Furthermore, we evaluated whether anti-fHbp antibodies were able to inhibit the binding of fH (using the highest fH concentration, 50 μg/ml) to the surface of each bacterial strain.

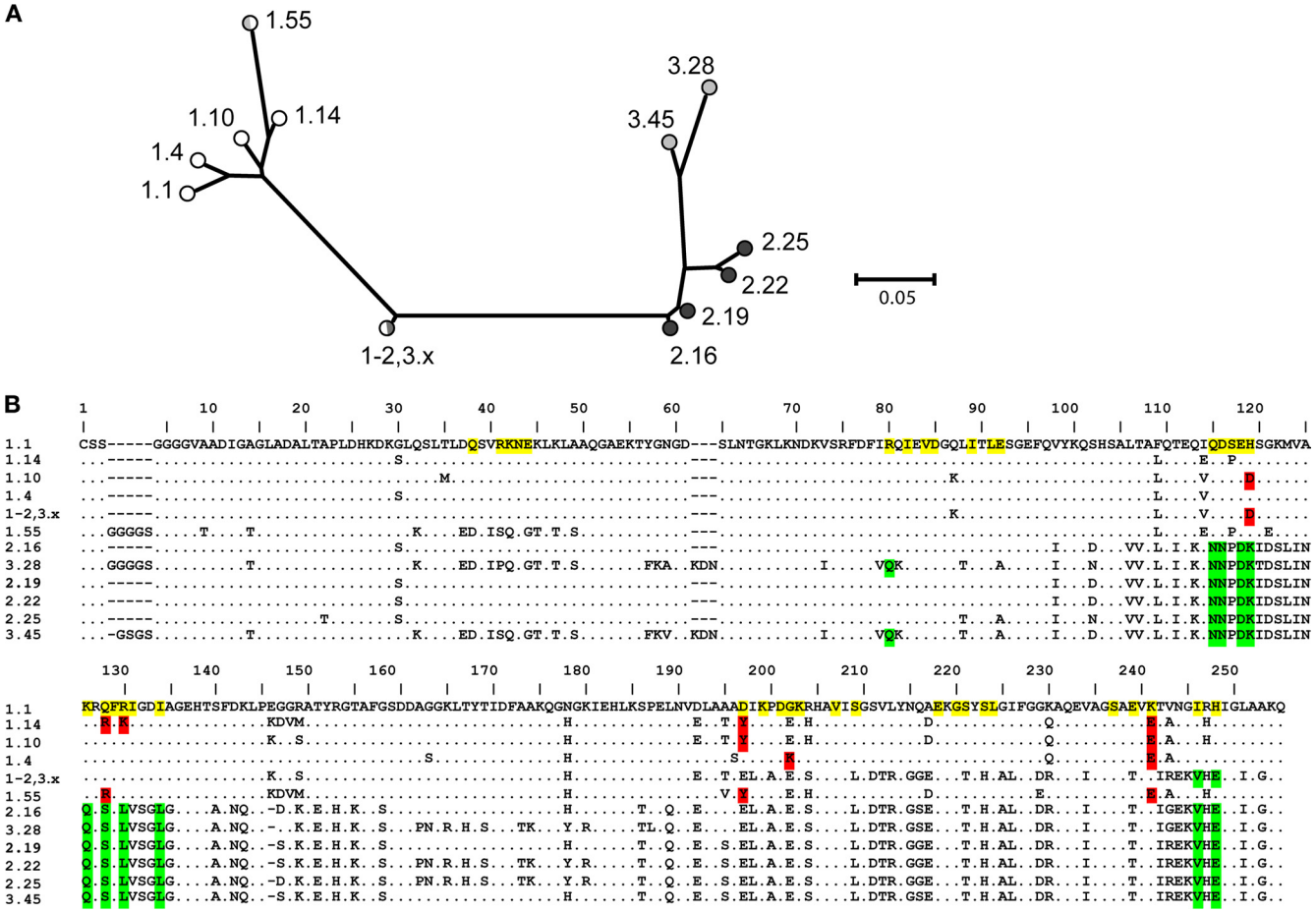

FIG. 1. Alignment and phylogenetic analysis of the fHbp subvariants. (A) Phylogenetic reconstruction of the 12 fHbp subvariants used in this study, based on the distance matrix obtained using the neighbor-joining method, where the tree branch lengths are proportional to the evolutionary distances used to infer the phylogenetic tree. Sequences belonging to fHbp variant groups 1, 2, and 3 are shown in white, dark gray, and light gray, respectively. fHbp 1-2,3.x and 1.55 are highlighted as chimeric subvariants. Based on measurements of the rate of amino acid mutations per position, the average distances of subvariants within the variant 1, 2, and 3 groups were 0.077 (±0.012), 0.041 (±0.010), and 0.070 (±0.016), respectively. The average distances between groups 1 and 2, groups 1 and 3, and groups 2 and 3 were 0.384 (±0.042), 0.470 (±0.046), and 0.123 (±0.019), respectively. fHbp 1-2,3.x is in an intermediate position between variants 1, 2, and 3, with distances of 0.174, 0.205, and 0.307, respectively. (B) Alignment of the amino acid residues of the fHbp subvariants, using subvariant 1.1 as a reference. Residues identical to subvariant 1.1 are shown as dots. Residues previously described to be involved in interaction with fH are shown in yellow on the 1.1 sequence. Amino acids typical of less stable fH binders (deleterious for affinity) are shown in red. Amino acids typical of more stable fH binders (potentially increasing the affinity) are shown in green (see Table 3). Except for subvariant 1.1, which was used as the reference sequence, the subvariants are ordered in the alignment by increasing fH affinity, according to the surface plasmon resonance dissociation rate constant (subvariants 1.1, 1.4, 1.10, 1-2,3.x, and 1.55 are classified as lower-affinity binders, while 2.16, 3.28, 2.19, 2.22, 2.25, and 3.45 are considered higher-affinity binders).

The results showed that antibodies against each of the homologous fHbp variants have the ability to decrease the binding of fH to the cell surface (Fig. 2C), demonstrating that in this panel of strains fHbp is responsible for the majority of fH binding.

MC58 strains engineered to express equivalent levels of fHbp subvariants differ in their level of fH binding. In order to investigate fH binding by each fHbp subvariant, independently of differences in expression level or strain genotype, an isogenic panel of strains was generated to express a subset of five of the fHbp variants described above. The isogenic panel consisted of the MC58Δ*fHbp* host strain, into which genes encoding different fHbp subvariants were inserted in *trans* under the control of the constitutive P_{tac} promoter, to enable similar levels of gene expression. Incubation of these strains with human fH, as described above, revealed that despite there being a similar level of fHbp expression between the strains (Fig. 3A), there was a different level of binding between the variants (Fig. 3B). The small differences seen in surface reactivities for the anti-fHbp sera were most likely a result of different immunogenicities of the proteins used to raise the mouse antisera and/or the differences in affinities of the antibodies used, rather than differences in expression. Subvariant 1.10 bound the least amount of fH, consistent with the data obtained with the strains naturally expressing this subvariant. Subvariants 1.1, 2.16, and 3.28 had medium-level binding, while subvariant 2.25 had the highest level of binding (relative to subvariant 1.1, the approximate relative levels of fH binding were 0.3 for subvariant 1.10, 0.9 for subvariants 2.16 and 3.28, and 2.6 for subvariant 2.25). These data suggest that while the ability of a strain to

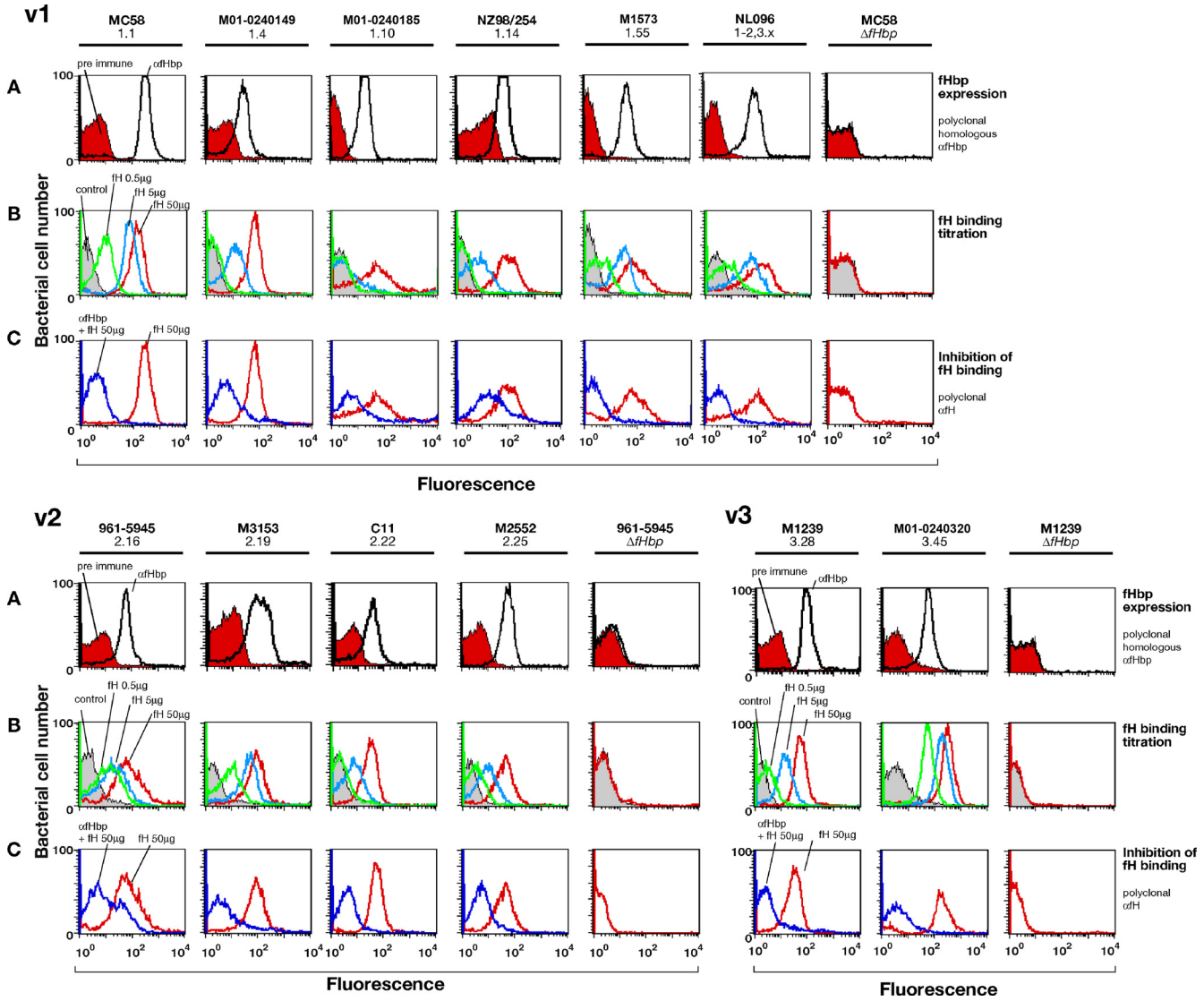

FIG. 2. Flow cytometry analysis of fHbp surface expression and fH binding of *N. meningitidis* strains. (A) Expression of fHbp on the meningococcal cell surface as detected by binding of polyclonal antiserum raised against the homologous fHbp subvariant for each strain. (B) Binding of human fH (0.5, 5, or 50 μg/ml) to the cell surface, detected by binding of polyclonal antiserum raised against human fH. (C) Inhibition of fH binding after incubation of bacteria with the polyclonal homologous fHbp antiserum. The strain name and the fHbp subvariant expressed by each strain are shown above panel A.

bind fH is influenced by the expression level of fHbp, as previously reported, differences in the sequences of the subvariants also affect fH binding.

Recombinant fHbp subvariants differ in their ability to bind human fH. SPR analysis was used to investigate the interaction between fH and the purified recombinant fHbp subvariants, in terms of binding affinity and association/dissociation kinetics. All fHbp subvariants bound fH, and the kinetics of association (k_a) with fH were comparable for all variants tested (within a range of ~3-fold above or below the k_a for subvariant 1.1) (Table 3). However, larger differences were seen in the dissociation kinetics (k_d), which describe the stability of the complex. The most stable binding to fH was observed by subvariants 2.25 and 3.45 (lowest k_d, approximately 10-fold lower than for subvariant 1.1), while the least stable fH binding was seen by subvariants 1.14 and 1.10 (highest k_d, approximately 10-fold higher than 1.1). The corresponding thermodynamic dissociation constants (K_D values) were in agreement with these differences (Table 3). The sensorgrams for subvariants 1.1, 1.10, and 2.25 are shown as representatives of the different stabilities of binding (Fig. 4). These results support the findings described above for fH binding by MC58 expressing different subvariants at equivalent levels, where subvariant 1.10 had the lowest binding affinity and 2.25 had the highest.

The differences seen in the kinetics analysis suggest that fHbp sequence variability influences the stability of binding between fH and fHbp. In principle, residues specifically found in subvariants with lower affinity for fH could be considered responsible for higher dissociation rates. On the contrary, residues specifically observed in subvariants with higher affinities

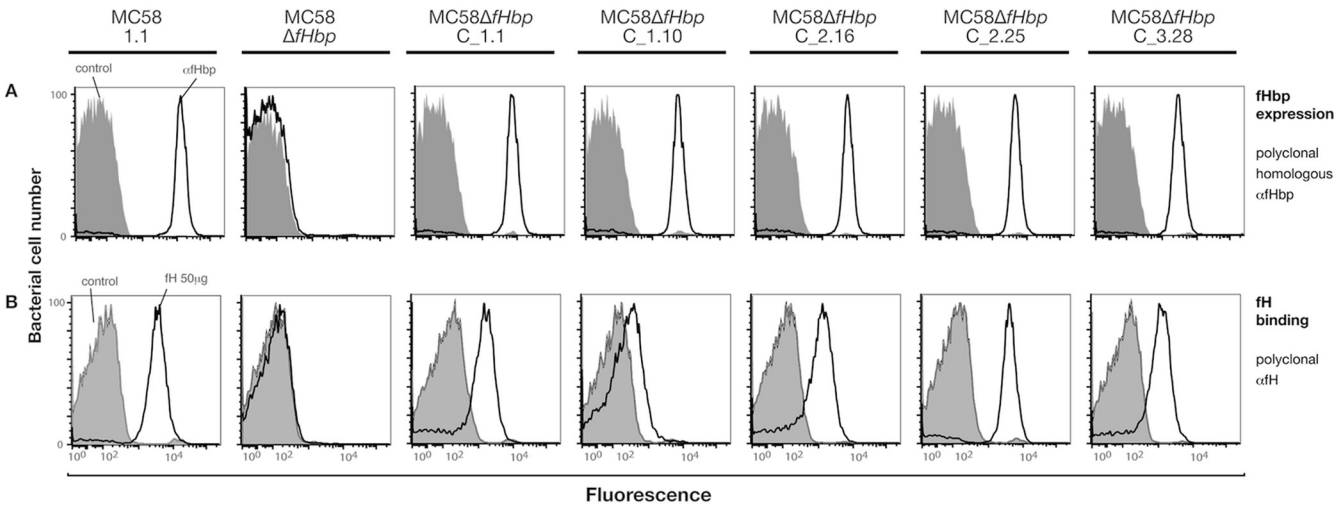

FIG. 3. Flow cytometry analysis of fHbp surface expression and fH binding of *N. meningitidis* MC58 strains engineered to express different fHbp subvariants. (A) Expression of fHbp on the meningococcal cell surface as detected by binding of polyclonal antiserum raised against the homologous fHbp subvariant for each strain. White profiles show reactions with immune sera. (B) Binding of human fH (50 μg/ml) to the cell surface, detected by binding of polyclonal antiserum raised against human fH (white profiles; gray profiles represent the negative control, to which no fH was added to the sample).

could increase the stability of the fHbp-fH complex. Although the sequence comparison of the different subvariants did not lead to an unambiguous identification of single amino acids responsible for increased or decreased affinities for fH, a collection of residues has been identified that could affect the stability of the complex (Fig. 1B).

Serum survival of MC58 strains expressing fHbp subvariants does not correlate with the level of fH binding. To determine if higher levels of fH binding lead to increased resistance to complement-mediated killing in human serum, MC58 strains expressing different fHbp subvariants were evaluated in an *ex vivo* human serum assay (Fig. 5). The MC58Δ*fHbp* strain is highly sensitive to killing by human serum due to the decreased binding of fH and thus the decreased inhibition of complement activation, as previously reported (26, 49). Resistance to serum killing was restored by complementation of this knockout with each of the five subvariants examined, but to different degrees. The subvariants 1.1 and 1.10 provided the greatest resistance, while subvariants 2.16, 2.25, and 3.28 displayed 3- to 20-fold-lower survival, depending on the serum donor. This suggests that there is not a direct correlation between the amount of fH bound to the bacterial surface and the level of resistance to complement-mediated killing. In fact, subvariant 1.10, which had the lowest level of fH binding, provided one of the highest levels of survival in serum to the recombinant MC58 strain.

TABLE 3. Kinetics of fH binding to fHbp subvariants based on surface plasmon resonance analysis

fHbp variant[a]	k_a (M^{-1} s^{-1})	k_d (s^{-1})[b]	Relative k_d[c]	K_D (nM)
1.1[I]	1.43×10^5	0.65×10^{-2}	1.0	45
1.4	3.38×10^5	1.86×10^{-2}	2.9	55
1.10	2.85×10^5	5.09×10^{-2}	7.8	178
1.14[II]	2.23×10^5	7.16×10^{-2}	11.0	350
1.55	0.76×10^5	0.78×10^{-2}	1.2	102
1-2,3.x	0.87×10^5	1.65×10^{-2}	2.5	190
2.16	0.96×10^5	0.42×10^{-2}	0.7	44
2.19	0.54×10^5	0.21×10^{-2}	0.3	40
2.22	0.45×10^5	0.22×10^{-2}	0.3	48
2.25[III]	0.60×10^5	0.04×10^{-2}	0.1	7
3.28	1.66×10^5	0.26×10^{-2}	0.4	16
3.45	0.59×10^5	0.09×10^{-2}	0.1	16

[a] See Table 1 for fHbp subvariant details. Replicate measurements for representative subvariants (shown with superscript roman numerals) were as follows: I, medium-affinity fH binder fHbp 1.1, k_a of $(1.43 \pm 0.21) \times 10^5$, k_d of $(0.65 \pm 0.13) \times 10^{-2}$, and K_D of 45 ± 5; II, low-affinity fH binder fHbp 1.14, k_a of $(2.23 \pm 0.28) \times 10^5$, k_d of $(7.16 \pm 1.39) \times 10^{-2}$, and K_D of 350 ± 115; III, high-affinity fH binder fHbp 2.25, k_a of $(0.60 \pm 0.08) \times 10^5$, k_d of $(0.04 \pm 0.01) \times 10^{-2}$, and K_D of 7 ± 0.5. *P* values, calculated using Student's *t* test, for comparisons of the other two representative subvariants to fHbp 1.1 were as follows: for fHbp 1.14, $P = 0.08$ for k_a, $P = 0.01$ for k_d, and $P = 0.06$ for K_D; for fHbp 2.25, $P = 0.06$ for k_a, $P = 0.03$ for k_d, and $P = 0.01$ for K_D.

[b] A lower k_d value corresponds to higher-affinity binding.

[c] The k_d of fH binding for each subvariant relative to subvariant 1.1.

fHbp subvariants induce bactericidal antibodies that differ in their levels of cross-protection within and between variant groups. The established correlate of protection for meningococcal disease is the serum bactericidal antibody (SBA) assay, which measures complement-mediated bacterial killing by serum antibodies. To evaluate the ability of each fHbp subvariant to induce bactericidal antibodies against MenB strains carrying homologous or heterologous subvariants, postimmunization sera from mice injected with each subvariant formulated with aluminum hydroxide were tested against 12 natural strains expressing the fHbp subvariants used in this study. As shown in Table 4, in SBA assays using rabbit complement, all subvariants induced functional bactericidal antibodies against most of the strains in the same variant group. Bactericidal titers were generally higher against strains carrying the homologous subvariant, with the exception of subvariant 1.55, which was negative for the homologous strain M1573. However, strain M1573 was highly resistant to killing by all polyclonal mouse antisera tested (this strain was sensitive to the anticapsular

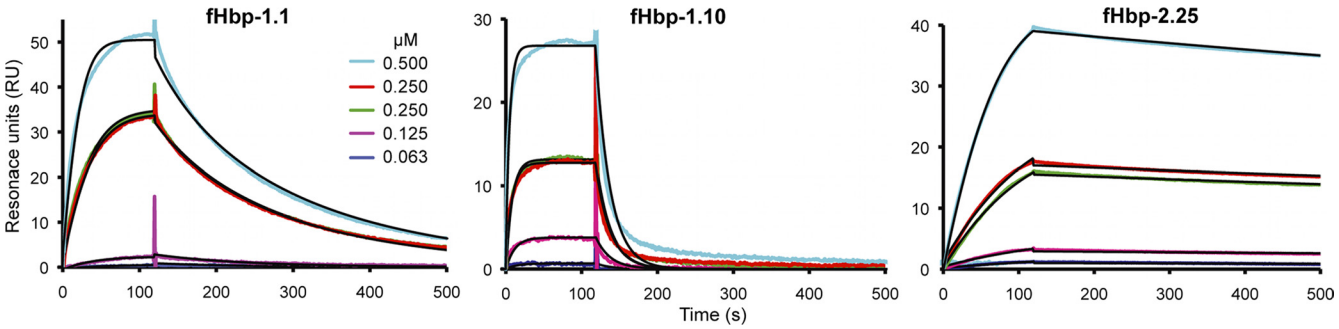

FIG. 4. Surface plasmon resonance kinetic analyses. Recombinant fHbp subvariants 1.1, 1.10, and 2.25 were flowed over the fH-bound sensor chip at different concentrations. Samples (0.250 μM; green and red lines) were run in duplicate. Interaction parameters were determined by a simultaneous local fitting (black lines) with a model of equimolar stoichiometry using the BIAevaluation X100 software version 1.0.

monoclonal antibody SEAM 12). However, when the anti-1.55 antisera was analyzed in the bactericidal assay against a recombinant MenB strain that was engineered to express the homologous subvariant, a bactericidal titer of 8,192 was obtained, suggesting that the negative titers against the homologous strain reflect an intrinsic resistance of this strain to SBA. fHbp 1.1, 1.4, 1.10, and 1.14 subvariants elicited high bactericidal titers against strains carrying fHbp from variant group 1. Similarly, fHbp 2.16, 2.19, 2.22, 2.25, and 3.28 elicited high bactericidal titers against strains expressing a variant 2 or 3 fHbp. As expected, there is some cross-coverage between groups 2 and 3 (26). However, subvariant 3.45 was less effective in terms of inducing sera able to provide cross-coverage. Subvariant 1.55, described as a chimeric form (Fig. 1), did not elicit bactericidal antibodies against MC58 (expressing fHbp 1.1), possibly because of the sequence distance between these two subvariants. In addition, it had no bactericidal activity against strains expressing variant 3 fHbp, suggesting that the region of sequence identity does not include bactericidal epitopes. In contrast, the 1-2,3.x chimera provided a particularly broad range of coverage against strains carrying each of the three variant groups, which was likely due to the more pronounced hybrid nature of the protein and the presence of conserved epitopes from all three groups. The SBA assays were also performed using human complement (Table 4) and, as expected, there was a general decrease in the bactericidal titers and the cross-reactivities seen. However, the trend of cross-coverage within the same variant and between variants 2 and 3 was maintained. Cross-coverage between the three variants was induced only by fHbp-1-2,3.x.

DISCUSSION

fHbp has been the focus of increasing interest in recent years due to its important role in meningococcal survival and species

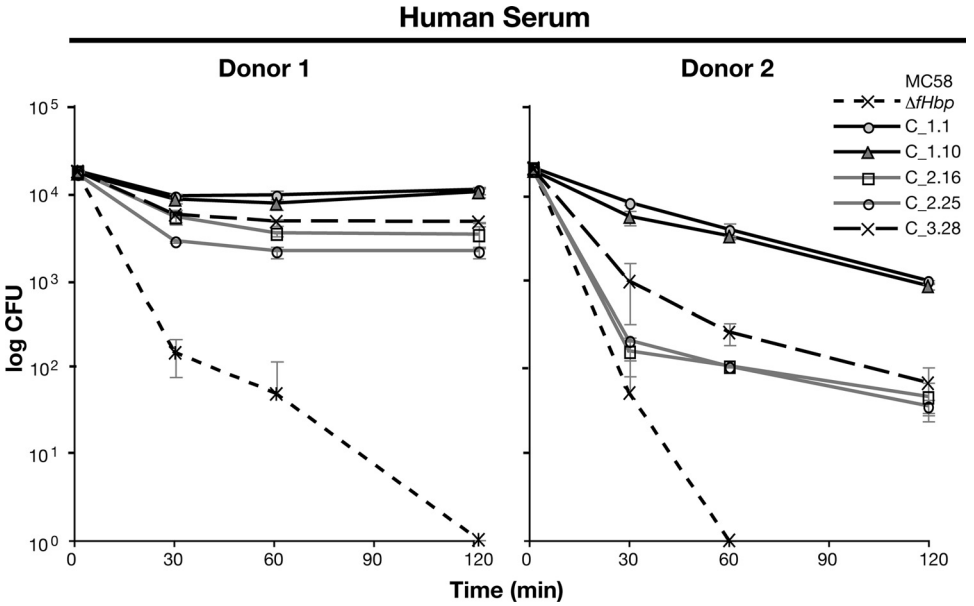

FIG. 5. Survival of *N. meningitidis* MC58 strains engineered to express fHbp subvariants in an *ex vivo* human serum model of meningococcal infection. Experiments were performed in triplicate on several occasions, and representative results are shown for sera from two different donors. Error bars indicate ±1 standard deviation of the mean. *P* values determined using Student's *t* test for survival at 120 min were as follows: between Δ*fHbp* and Δ*fHbp*C_1.1 (C_1.1), $P < 0.004$; for subvariants in group 1 compared to subvariants in group 2, $P < 0.02$; between subvariants within group 1 or within groups 2 and 3, $P > 0.1$.

TABLE 4. Serum bactericidal activity induced by fHbp subvariants

Complement source and *N. meningitidis* strain (fHbp subvariant)	Bactericidal titer[a] for anti-fHbp sera against:											
	Variant 1				Chimeric		Variant 2				Variant 3	
	1.1	1.4	1.10	1.14	1.55	1-2,3.x	2.16	2.19	2.22	2.25	3.28	3.45
Rabbit complement												
MC58 (1.1)	[**>8,192**]	**>8,192**	**4,096**	**>8,192**	<16	**>8,192**	<16	64	<16	16	64	<64
M01-0240149 (1.4)	**512**	[**2,048**]	**1,024**	**1,024**	**1,024**	**2,048**	64	<16	<16	<16	<16	<32
M01-0240185 (1.10)	**256**	**128**	[**2,048**]	**2,048**	**1,024**	**2,048**	<16	<16	<16	<16	<16	<16
NZ98/254 (1.14)	**128**	**512**	**2,048**	[**>8,192**]	**256**	**512**	<16	32	<16	<16	<16	<16
M1573 (1.55)	<16	<16	<16	<16	[<16[b]]	<16	<16	<16	<16	<16	<16	<16
NL096 (1-2,3.x)	<16	32	<16	<16	<16	[**8,192**]	<16	<16	<16	<16	<16	<16
961-5945 (2.16)	32	**128**	64	**256**	<32	**>8,192**	[**>8,192**]	**>8,192**	**2,048**	**2,048**	**4,096**	**512**
M3153 (2.19)	<16	32	**128**	**256**	<16	**2,048**	**8,192**	[**8,192**]	**2,048**	**1,024**	**1,024**	64
C11 (2.22)	16	<16	<16	<16	<16	**128**	**256**	**2,048**	[**4,096**]	**256**	**2,048**	<16
M2552 (2.25)	16	<16	<16	<16	<32	**128**	**512**	**4,096**	**8,192**	[**512**]	**128**	<32
M1239 (3.28)	<16	<16	<16	<16	<32	**128**	**512**	**1,024**	**2048**	**512**	[**8,192**]	64
M01-0240320 (3.45)	<32	<32	<32	<32	<32	**2,048**	**2,048**	**>8,192**	**1,024**	**4,096**	**4,096**	[**4,096**]
Human complement												
MC58 (1.1)	[**2,048**]	**1,024**	**16**	**16**	<8	**32**	<8	<8	<8	<8	<8	<8
M01-0240149 (1.4)	**128**	[**1,024**]	**256**	**1,024**	<8	**256**	<8	<8	<8	<8	<8	<8
M01-0240185 (1.10)	<8	**512**	[**1,024**]	**512**	**512**	**512**	<8	<8	<8	<8	<8	<8
NZ98/254 (1.14)	**64**	**32**	**256**	[**1,024**]	**256**	**128**	<8	<8	<8	<8	<8	<8
M1573 (1.55)	<8	<8	<8	<8	[<8[b]]	<8	<8	<8	<8	<8	<8	<8
NL096 (1-2,3.x)	<8	<8	<8	<8	<8	[**1,024**]	<8	<8	<8	<8	<8	<8
961-5945 (2.16)	<8	<8	<8	<8	<8	**32**	[**2,048**]	**512**	**16**	<8	<8	**16**
M3153 (2.19)	<8	<8	<8	<8	<8	**256**	**128**	[**512**]	**64**	**128**	**32**	<8
C11 (2.22)	<8	<8	<8	<8	<8	**64**	**128**	**128**	[**512**]	<8	**256**	<8
M2552 (2.25)	<8	<8	<8	<8	<8	<8	**128**	**128**	**128**	[**128**]	<8	<8
M1239 (3.28)	<8	<8	<8	<8	<8	<8	<8	<8	<8	<8	[**512**]	<8
M01-0240320 (3.45)	<8	<8	<8	<8	<8	**512**	**512**	**512**	**256**	**512**	**256**	[**2,048**]

[a] Bactericidal titers are expressed as the reciprocal of the serum dilution of mouse polyclonal antibodies yielding ≥50% bactericidal killing. Antigens were formulated in aluminum hydroxide, and assays were performed with rabbit complement (rSBA) or human complement (hSBA); rSBA titers of ≥128 and hSBA titers of ≥16 (4-fold increase above baseline) are shown in boldface. The values in brackets show the SBA titer obtained for each fHbp subvariant versus the strain expressing the homologous subvariant.

[b] A bactericidal titer of 8,192 was seen for subvariant 1.55 when we used a recombinant MenB strain that was engineered to express the homologous subvariant, demonstrating its immunogenicity. Negative results were obtained for strain M1573, which was highly resistant to killing by all antisera.

specificity, as well as its inclusion in two investigational MenB vaccines currently in clinical trials (reviewed in reference 38). Despite fHbp having a conserved function in *N. meningitidis*, with fH binding providing the ability to downregulate killing by the human complement pathway, several genetically and antigenically diverse variants of fHbp have been identified (1, 31). It has been proposed that these variants have a mosaic architecture, like many meningococcal proteins, that may result from the high degree of horizontal gene transfer that is common in pathogenic *Neisseria* (2, 31). In this study we characterized a selection of fHbp subvariants in terms of their expression, ability to bind fH, role in serum resistance, and their immunogenicity and ability to induce cross-protection in SBA assays, in order to better understand the functional significance of fHbp sequence variability. This selection of subvariants included fHbp 1.1, which is present in the Novartis MenB vaccine, as well as fHbp 1.55 (B01) and fHbp 3.45 (A05), which are present in the Pfizer MenB vaccine. Two particular chimeric forms of fHbp were present in the panel of subvariants analyzed, i.e., 1-2,3.x and 1.55.

Through a detailed analysis of fH binding to 12 different subvariants, using several approaches with live bacteria (natural and recombinant strains) or purified recombinant proteins, we have demonstrated that different fHbp subvariants have different fH binding characteristics. In particular, we found that a panel of natural strains, or a single recombinant strain expressing various fHbp subvariants, bind different levels of fH. Differences in the levels of binding of fH to the bacterial surface did not always correlate with the expression level of fHbp, but rather, the stability of binding seems to be an important factor. SPR analysis of fH binding to recombinant fHbp proteins showed a range of binding affinities between the subvariants that varied over 2 orders of magnitude for the dissociation rate constant (k_d) and more than 1 order of magnitude for the dissociation constant (K_D). These results are supported by the findings on a different panel of subvariants that were published by Dunphy et al. (7) while our manuscript was under review. It has been elucidated that fHbp binds to short consensus repeat 6 (SCR6) of fH (50). Moreover, the nuclear magnetic resonance structure of the C-terminal portion of fHbp 1.1 (5) and the crystal structure of fHbp 1.1 in complex with fH SCR67 (48) have led to identification of residues important for binding of anti-fHbp antibodies and fH, respectively. High-affinity binding was previously seen between the fH SCR67 fragment and subvariant 1.1 (K_D of 5 nM) (48). We also saw high-affinity binding in our experiments when we used the entire fH molecule (SCR1-20), with a K_D of 45 nM for subvariant 1.1, while the K_D values of the subvariants ranged from 7 to 350 nM. This variation in binding affinity was evident via flow cytometry using whole bacteria and titration of the fH

concentrations, with some variants binding even at the very low concentrations of fH (0.5 μg/ml), while others showed no binding. The most variable residues between the three variant groups, as well as those involved in fH binding or those that are targeted by monoclonal antibodies, were found to be in the upper exposed surface of the molecule (C domain), which most likely has the greatest exposure to the immune system (5, 48). It has been proposed that an extended recognition site for fH exists across the entire surface of fHbp, with numerous electrostatic interactions, H bonds, and salt bridges with both β-barrels of fHbp (48). Twenty amino acid residues of fHbp have been identified that interact with fH, and substitution of two glutamate residues led to a loss of fH binding (48). Since one of these glutamate residues is not conserved in all the variants analyzed in this study, we speculate that other residues may be involved in fH binding. Indeed, there are several amino acid residues that are conserved among the subvariants with higher binding affinities. Residues specifically present in higher- or lower-affinity binders tend to cluster in three regions that contain residues known to be involved in interaction with fH (48), namely, residues 115 to 119, 126 to 134, and 241 to 248. In particular, residues 119, 128, and 130 have substitutions that are associated either with higher-affinity binding (H119/K, Q128/S, and R130/L) or lower-affinity binding (H119/D, Q128/R, and L130/K) to fH. In all of these cases, a difference in charge was observed between the sequences of stable and less stable binders, confirming the hypothesis that electrostatic effects play a critical role in modulating the interaction of fHbp with fH. Further sequence comparisons and analyses of binding capabilities of mutants may help confirm the roles of these residues.

In terms of the functional significance of the different levels of fH binding displayed by the subvariants and strains, the level of serum resistance of a strain did not correlate with the level of fH binding or the affinity of binding. This was particularly evident for *N. meningitidis* strain MC58Δ*fHbp* complemented with subvariant 1.10; this strain had one of the lowest levels of fH binding (~3-fold lower than to subvariant 1.1) but had equivalent survival to the strain complemented with subvariant 1.1. Subvariant 1.10 also had one of the lowest binding stabilities in SPR analyses. It is important to note that fH is present at high levels in serum (110 to 615 μg/ml, or 0.71 to 3.9 μM [42]), which is in excess of the concentrations used in these studies, and binding of fH to fHbp is most likely saturated even for strains with subvariants with low binding affinity or stability. However, fH is also present in other sites relevant to meningococcal colonization and disease, including the nasopharynx (36, 54) and the cerebrospinal fluid (CSF) (16). The levels of fH in these sites have not been well characterized, but concentrations at mucosal surfaces are expected to be ~10% of serum levels, and it has been reported that levels in CSF are <1% of those found in serum (16). Hence, the different levels of fH binding by subvariants may be more relevant in different niches during colonization or disease, rather than during disease in the blood. Also, it has been reported that for strain 2996, which has very low fHbp expression and negligible fH binding, that deletion of the *fHbp* gene leads to increased serum sensitivity (25). These data suggest that either low levels of fH binding are sufficient to provide serum resistance and/or that other host factors also play a role in fHbp-dependent survival in human serum. Indeed, *N. meningitidis* Δ*fHbp* mutant strains (both subvariants 1.1 and 1.10) have been shown to be sensitive to killing by the antimicrobial peptide LL-37, indicating an additional role of fHbp (49). This may be relevant for survival in serum, given that the precursor of LL-37, hCAP-18, is present in plasma at levels of approximately 1.2 μg/ml, which is more than 20% of the amount in circulating neutrophils (51). The importance of various factors of the immune system in preventing meningococcal disease is indicated by the increased incidence of disease in people with deficiencies of fH or complement factors (i.e., factor I or C5 to C9) (15, 27, 34, 40). Work is under way to determine if different subvariants mediate different levels of resistance to LL-37 and/or additional host defenses. It was recently reported that neisserial surface protein A (NspA) also binds fH, particularly in unencapsulated strains expressing lipopolysaccharide with truncated heptose I chains, and it enhances meningococcal resistance to complement (22), indicating that additional factors may influence the level of fH binding by the panel of strains examined. Since more than one meningococcal protein can interact with fH, it is possible that a specific fHbp subvariant may be best suited to a particular strain if it has evolved with a certain combination of protein variants to ensure optimal fitness in the host. This could be relevant to the level of serum resistance seen for the MC58 strains engineered to express different subvariants; for example, complementation of MC58 (which normally expresses fHbp 1.1) with a subvariant 2 or 3 protein may be less effective in mediating serum resistance than complementation with a more closely related subvariant 1 protein.

In terms of the immunogenicity of fHbp and its ability to induce bactericidal antibodies in mice, the subvariants analyzed displayed different levels of cross-reactive bactericidal activity within each variant group in the panel of strains tested in SBA assays using both rabbit and human complement. The majority of subvariants were unable to induce antibodies that were cross-protective between the three variant groups, supporting previously published data (26). However, of particular interest was the finding that the natural 1-2,3.x chimera induced high SBA titers against subvariants in each of the three variant groups. This broad coverage is likely due to the hybrid nature of the protein sequence and the presence of epitopes from the variant 1, 2, and 3 groups. However, subvariant 1.55 is also a hybrid between fHbp 1and fHbp 3, but a similar broad protection was not seen by this subvariant under our experimental conditions. The lower titers and cross-reactivity seen in the SBA assays using human complement may be due to the interactions of human fH with the meningococci and the downregulation of complement activation. However, we have shown that binding of anti-fHbp antibodies to meningococcal strains decreases the amount of fH binding to the bacterial surface, and therefore it is not expected that complement activation would be fully downregulated in the presence of human complement and anti-fHbp antibodies. It has been suggested that the best predictor for killing by anti-fHbp antibodies is the surface expression level of fHbp (26). However, the variability of cross-reactive bactericidal activities within a variant group suggests that amino acid variations within each subvariant also have an important influence on the bactericidal activity.

Many microbial surface proteins, in addition to fHbp, have been identified that have extensive sequence variability and yet

retain a conserved ligand binding function, for example, C4BP binding by the hypervariable region of *Streptococcus pyogenes* M protein, sialic acid binding by hemagglutinin (HA) of influenza virus, and CD4 binding by gp120 of HIV-1. HA and gp120 both have regions that are relatively well conserved between variants that are required for ligand binding (6, 33, 56), whereas M protein variants lack conserved amino acid motif or residues to explain binding (37). *N. meningitidis*, as a strictly host-adapted pathogen, is adept at varying its surface structures to avoid host defenses (30). As such, fHbp seems to have evolved to maintain conserved regions that are involved in fH recognition and enable activation of the alternative complement pathway to be decreased at the bacterial surface but also contain a large degree of variability, which may enable the bacteria to escape classical complement activation.

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